Revised Manuscript for ***Bioscience, Biotechnology, and Biochemistry***

Extracts of bilberry (*Vaccinium myrtillus* L.) fruits improve liver steatosis and injury in mice by preventing lipid accumulation and cell death

Sanae Haga[a], Yimin[b], Hikari Yamaki[a], Shigeki Jin[c], Tetsuya Sogon[d], Naoki Morita[e] and Michitaka Ozaki[a,f]

[a]Department of Biological Response and Regulation, Faculty of Health Sciences, Hokkaido University, N12W5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan; [b]Department of Advanced Medicine, Graduate School of Medicine, Hokkaido University, N12W5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan; [c]Department of Forensic Medicine, Graduate School of Medicine, Hokkaido University, N15W7, Kita-ku, Sapporo, Hokkido 060-8638, Japan; [d]WAKASA SEIKATSU Corporation, 22 Naginatabokocho, Shijokarasuma, Shimogyo-ku, Kyoto, Kyoto 600-8008, Japan; [e]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan; [f]Laboratory of Molecular and Functional Bio-imaging, Faculty of Health Sciences, Hokkaido University, N12W5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

CONTACT

Naoki Morita, Ph.D.

Address: Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST),

2-17-2-1 Tsukisamu-higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan

Tel.: +81-11-857-8963

Fax: +81-11-857-8992

E-mail address: morita.n@aist.go.jp (N. Morita)

Michitaka Ozaki, M.D., Ph.D.

Address: Department of Biological Response and Regulation, Faculty of Health Sciences, Hokkaido University,

N12W5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

Tel.: +81-11-706-3337

Fax: +81-11-706-3337

E-mail address: ozaki-m@med.hokudai.ac.jp (M. Ozaki)

KEYWORDS

Bilberry fruits; fatty liver; liver injury; non-alcoholic steatohepatitis (NASH); *Vaccinium myrtillus*

Supplementary data for this article can be accessed https://doi.org/XXXXX

ABSTRACT

Bilberry has been reported to have anti-oxidant and anti-inflammatory properties. We studied the effect of bilberry (*Vaccinium myrtillus* L.) fruits extracts (BEs) on the pathogenesis caused by lipid accumulation in fatty liver and non-alcoholic steatohepatitis (NASH). 5 μg/ml of BEs was enough to suppress lipid accumulation in the fatty liver model of the mouse hepatic AML12 cells. BEs increased cell viability and anti-oxidant capacity, presumably by activating (phosphorylating) Akt/STAT3 and inducing MnSOD/catalase. BEs also significantly reduced Rubicon and induced p62/SQSTM1, possibly contributing to reduce cellular lipids (lipophagy). When the mice were fed supplemented with BEs (5% or 10%, w/w), hepatic steatosis, injury, and hypercholesterolemia/hyperglycemia were significantly improved. Furthermore, histological and cytokine studies indicated that BEs possibly suppress hepatic inflammation (hepatitis) and fibrosis. Therefore, BEs improved liver steatosis and injury, and potentially suppress fibrosis by suppressing inflammatory response, which therefore may prevent the progression of fatty liver to NASH.

The perennial dwarf shrub bilberry (*Vaccinium myrtillus* L.) is also known as the European blueberry and is widely grown in the northern hemisphere across Europe and Central Asia [1]. Although bilberry leaves are a promising source of bioactive natural products [2], bilberry fruits are better known for the health benefits of its phytochemicals. Bilberry fruits are rich in anthocyanidins and anthocyanins [3] and other potent natural antioxidants like flavanols and phenolic compounds. Several studies have shown that *V. myrtillus* berries contain higher anthocyanin and antioxidant levels than cultivated blueberries (*V. corymbosum*) [4].

Wild bilberry fruits are well established in pharmacognosy and have been used as a food source for centuries. Bilberry is a rich source of anthocyanins [3] and its extracts are extensively used in dietary supplements and pharmaceutical products. Bilberry fruits extracts are antioxidant [5-8], can decrease capillary permeability and fragility [9], inhibit platelet aggregation [10], and strengthen collagen matrix cross-linkages [11,12]. Bilberry crude extracts are used in the treatment of ophthalmological diseases and blood vessel disorders [13]. Recently, they have been administered to treat dysentery, diarrhea, and mouth and throat inflammations [14]. Certain studies have shown that the bilberry anthocyanins may prevent metabolic syndrome [15-22]. However, the mechanisms by which bilberry influences diabetes and lifestyle-related diseases have not yet been identified. Since bilberry seems to prevent diabetes mellitus, it may also prevent non-alcoholic steatohepatitis (NASH), which is a progressive and severe form of non-alcoholic fatty liver disease (NAFLD). Fat accumulation in liver is involved in the onset and progression of these conditions [23,24].

In the present study, we investigated the effects of dried bilberry (*Vaccinium myrtillus)* fruits extracts (BEs) especially on mouse hepatic steatosis and injury in cellular and animal models. We elucidated the possible mechanisms of BEs on the prevention of fatty liver and NASH, with regards to hepatic steatosis and injury. Also, we proposed the possible molecular mechanisms of BEs effects on the prevention of fatty liver and NASH, regarding autophagy, fat metabolism, oxidative stress and mitogenesis.

Materials and methods

Cell culture, reagents and bilberry fruits extracts (BEs)

Alpha mouse liver 12 cells (AML12; ATCC® CRL-254™, Manassas, VA, USA) were maintained at 37 °C and 5.0% CO_2 in DMEM/Nutrient Mixture F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) and 1× ITS-A (Insulin-Transferrin-Selenium-Sodium Pyruvate Supplement, Thermo Fisher Scientific, Waltham, MA, USA). Fatty acids (FA; oleic and linoleic) and T0901317 (T090; LXRα agonist) were purchased from TCI Chemicals (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. To induce steatosis of hepatocytes, T090 (1 μM) or FA (100 μM for each oleic and linoleic acids) were added to the culture media daily for 3 or 5 days [25-27].

A 90% ethanolic extracts of bilberry (*Vaccinium myrtillus* L.) fruits was purified with an adsorbent resin to obtain bilberry fruits extracts containing ≥36% anthocyanin glycosides. The purified product was lyophilized, homogenized, and used as bilberry fruits extracts (BEs). BEs were added daily to the culture media at the designated concentration. We used BEs in the concentrations from 1 μg/ml to 10 μg/ml, which was proved to show no cytotoxicity to AML12 hepatocytes by lactose dehydrogenase (LDH) assay using LDH Cytotoxicity Detection Kit (Takara Bio Inc., Shiga, Japan) (Figure S1).

Hepatic lipid accumulation analysis

AML12 cells were rinsed with PBS, fixed with 4% paraformaldehyde/PBS for 5 min, and stained with Nile red (AdipoRed Assay Reagent, Lonza, Basel, Switzerland). After incubation at room temperature for 10-15 min, cells were quantified and observed for fluorescence with excitation at 485 nm and emission at 572 nm (expressed as relative fluorescence units, RFU) in a multimode plate reader (Infinite® 200 PRO, TECAN, Zurich, Switzerland) and under a

fluorescence microscope (Biozero; Keyence, Osaka, Japan). Hoechst33342 (Sigma-Aldrich) was used for nuclear counterstaining. Relative lipid accumulation (RLA) was determined as the ratio of AdipoRed to Hoechs33342 intensity values to calculate well-level normalization. For all doses, the average RLAs of the technical replicates were calculated and normalized to vehicle control wells to determine the mean fold-change values across the test plates. The mean dose-response and standard error were derived from eight independent experiments.

For animal experiments, liver triacylglycerol (TG) contents were measured with an adipogenesis colorimetric/fluorometric assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions [28]. The TG concentration was calculated from a standard curve and normalized to mass per μg protein.

Monitoring and evaluation of hepatocyte growth (survival and proliferation)

The effects of BEs on the growth (survival/proliferation) of non-steatotic or steatotic AML12 cells were determined with the xCELLigence System (Roche, Basel, Switzerland). The survival/proliferation analyses of non-steatotic and steatotic hepatocytes were performed in the following way. Briefly, for the non-steatotic hepatocytes, untreated AML12 cells were plated at a density of 1.5×10^4 cells/well in an E-Plate 16 PET (ACEA Biosciences, San Diego, CA, USA), and for the steatotic hepatocytes, pre-steatotic AML12 cells were plated at a density of 1.2×10^3 cells/well in the plate. The plates were then placed in the xCELLigence System and BEs were administered daily at the designated concentrations. The above-mentioned experiments were monitored by real-time quantitation.

Evaluation of cellular redox states in vitro

Cellular redox states were imaged/evaluated with fluorescence microscopy using Redox-sensitive roGFP as previously described [29].

RNA extraction and qRT-PCR

Total hepatocyte RNA was isolated with an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The quantitative real-time PCR (qRT-PCR) was performed on the Bio-Rad CFX Connect System with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The used primers were listed in Table 1. The qRT-PCR data were analyzed by the comparative Ct Method ($2^{-\Delta\Delta Ct}$) [30]. The mRNA expressions were normalized to *36B4* (acidic ribosomal phosphoprotein P0 (RPLP0)) mRNA and the relative mRNA expression levels were calculated using the control mRNA.

Animal care, experimental design, and sample preparation

Male homozygous leptin receptor-deficient (BKS.Cg-+ Leprdb/+ Leprdb/Jcl; db/db) mice (10 weeks) were obtained from CLEA Japan (Tokyo, Japan). The mice were divided into four groups. Group 1 received a normal control diet. Group 2 received a high-fat and high-cholesterol (HF&HC) diet. Groups 3 and 4 received HF&HC diet supplemented with 5% and 10% BEs, respectively. The HF&HC diet (No. D09100301) was purchased from Research Diet Inc. (New Brunswick, NJ, USA), mixed with BEs, and freshly prepared every 2 days.

The mice were sacrificed before BEs administration and after 8 weeks to collect liver and blood specimens. The liver/body weight ratios were calculated to estimate relative changes in liver mass. Liver specimens were prepared for histological and western blot analyses. Blood specimens were used in biochemical analyses (alanine transaminase/glutamate pyruvate transaminase (ALT); aspartate transaminase/glutamate oxaloacetate transaminase (AST); glucose (GLU); and total cholesterol (T-CHO). The animal care and experimental procedures were approved by the Animal Research Committee of Hokkaido University (Permission No. 16-0120) and performed in strict accordance with the

Guidelines for the Care and Use of Laboratory Animals of Hokkaido University. All efforts were made to minimize animal suffering.

For histological analysis, liver specimens were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) or Azan. To visualize hepatic lipid accumulation, frozen sections of formalin-fixed liver tissue were stained with Sudan III. Briefly, frozen liver sections (8 μm thick) were mounted on slide glasses, air-dried, and rinsed with 50% v/v ethanol. Next, the specimens were stained in Sudan III stain for 10 min at room temperature and rinsed with 50% (v/v) ethanol to remove excess stain. Hematoxylin nuclear counterstain was applied for 3 min. The stained liver sections were then washed several times in water, mounted with a coverslip, and viewed under a microscope.

Immunoassay

For expression analysis of proteins associated with autophagy (lipophagy) and fatty acid (FA) synthesis, antibodies against the following proteins were used; Rubicon, p62/SQSTM1 (Cell Signaling Technology, Danvers, MA, USA), FASN (fatty acid synthase, BD Transduction Laboratories, Franklin Lakes, NJ, USA) and GAPDH (Cell Signaling Technology, Danvers, MA, USA) using standard western blot analysis protocol.

For expression analysis of proteins associated with autophagy, survival/proliferation, and antioxidant properties, protein separation and detection were performed using an automated capillary electrophoresis system, Wes™ (ProteinSimple, San Jose, CA, USA) and antibodies against the following proteins were used; p62/SQSTM1, phospho-Akt, Akt, phospho-STAT3 (Tyr705) (Cell Signaling Technology, Danvers, MA, USA), STAT3 (Santa Cruz Biotechnology, Dallas, TX, USA), manganese-dependent superoxide dismutase (MnSOD) (BD Transduction Laboratories, Franklin Lakes, NJ, USA), catalase (EMD Biosciences, Darmstadt, Germany), and GAPDH. Signals were detected with an HRP-conjugated secondary anti-rabbit or anti-mouse antibody, and were visualized using

Compass for ProteinSimple software.

Statistical analysis

All results were expressed as means ± standard error of the mean (SEM). ANOVA followed by Tukey–Kramer test were performed to compare the means. Statistical difference at $p < 0.05$ were considered as significant.

Results

Effects of BEs on hepatic cells

To determine the effects of BEs on hepatocyte steatosis, we created a steatotic murine hepatocytes in 2 ways by adding fatty acids (FA; oleic and linoleic) or a nuclear receptor LXRα agonist (T090) to AML12 cells. Figure 1A shows lipid accumulation induced by FA or T090, which are standardized by untreated AML12 cells (left panel). Fluorescence photomicrographs of cells showing lipids (green color) and nuclei (red color) are shown in Figure 1A (right panel). Both FA and T090 induced lipid accumulation in AML12 cells, though FA was more efficient. Cell growth (survival/proliferation) of the FA- and T090-induced steatotic AML12 cells was significantly lower than that of the untreated controls (Figure 1B). The addition of FA and T090 both inhibited the proliferation of AML12 cells, but the inhibitory effect of FA was observed at an early stage of proliferation, whereas that of T090 was observed at a later stage of proliferation. However, steatosis was significantly improved by adding BEs into the culture media. This effect of BEs was most apparent at 5 μg/mL BEs, indicating an optimum concentration for this effect (Figure 1C). BEs seem to suppress cellular lipid accumulation by both types of hepatic steatosis (FA and T090) dose-dependently (Figure 2A).

We examined the pro-survival effects of BEs in non-steatotic AML12 cells. BEs significantly promoted cell survival/proliferation showing a peak at 1 μg/mL (Figure 2B).

Oxidative stress (H_2O_2, 500 μM) reduced cell survival in non-steatotic AML12 cells, which was improved significantly by the treatment with BEs (Figure 2C). These results indicate that BEs confer a pro-survival property and a resistance against oxidative stress to hepatocytes. Therefore, we monitored changes of reactive oxygen species (ROS) levels over time in AML12 cells treated with H_2O_2. Interestingly, there were no significant differences between cells added with and without BEs in terms of their cellular ROS levels (Figure 2D). These data may indicate that BEs do not directly eliminate intracellular ROS, but BEs provide the resistance against oxidative stress. Although Nrf2, activated by oxidative stress and toxic substances in the environment, is well known to protect cells from environmental stresses including ROS, significant activation of an antioxidative Nrf2-DNA binding was not observed (data not shown).

Effect of BEs on mouse with fatty liver

Next, we challenged BEs against mouse fatty liver model to confirm the BEs' *in vivo* effects. HF & HC diet markedly increased liver/body weight ratios, hepatic steatosis/TG contents, and injuries, which were suppressed by BEs in a dose-dependent manner (Figures 3A, 3B, 3D). There was no significant difference in dietary intake between the groups receiving BEs and those not receiving them (Table 2). Histological study and hepatic TG measurement revealed that BEs administration undoubtedly suppressed fat accumulation/TG contents in mouse liver (Figure 3B). The HF & HC diet caused mild fibrosis around portal area in mouse livers (Figure 3C, arrowhead), whereas those being administered BEs presented much less fibrosis. Blood biochemistry of the mice fed with the normal and HF & HC diet are shown in Figure 3D. Plasma ALT and AST levels in the 5% BEs group were significantly lower than those in the group receiving HF & HC diet alone ($p < 0.05$). Plasma GLU and T-CHO levels for the 10% BEs group were also significantly lower than those for the HF & HC diet group. Furthermore, compared with those given the normal diet, a series of pro-inflammatory

cytokine levels (e.g. TNF-α, IL-9, IL-1β and IFN-γ) were higher in mice receiving the HF & HC diet, which were likely to be reduced by BEs administration though no significant difference was found among them (Figure S2).

These data indicate that BEs improve hyperglycemia/hypercholesterolemia, liver injury and inflammation, and presumably liver fibrosis.

Effects of BEs on the expression levels of the genes and proteins regulating lipid metabolism, survival/proliferation, antioxidant, and autophagy in hepatocytes

The mouse fatty liver model clearly showed that BEs improve fat accumulation, injury, inflammatory reaction, and fibrosis of liver. To elucidate the underlying molecular mechanisms, we examined the expressions of the genes and proteins related to these events using mouse AML12 cells.

Figure 4A shows the protein expressions of autophagy- and fat metabolism-associated proteins including Rubicon (run domain beclin-1-interacting and cysteine-rich domain-containing protein, a cellular protein that suppresses late stage of autophagy), p62/SQSTM1 (an autophagy marker), and FASN (fatty acid synthase) [31-34] in AML12 cells. Fat accumulation in hepatocytes was stimulated by the addition of FA and T090 to the culture media (Figure 1A). When AML12 cells were subjected to FA, Rubicon expression was dramatically elevated. The addition of BEs downregulated Rubicon significantly and concurrently upregulated p62/SQSTM1 expression significantly (Figure 4A, upper panel). Therefore, it is suggested that BEs enhanced autophagy (lipophagy) by suppressing Rubicon expression, and eventually reduced lipid accumulation. T090 clearly upregulated FASN, which was certainly suppressed by the addition of BEs. On the contrary, FASN was neither induced in FA-treated AML12 cells, nor affected by BEs administration at all (Figure 4A, upper panel). Taken together, BEs may improve fat accumulation by

enhancing autophagy (lipophagy) or suppressing FA synthesis in the steatotic hepatocytes, depending on the causes of steatosis.

We then studied the expression of genes related to FA synthesis including *LXR* (a member of the family of nuclear receptor transcription factors involved in lipid synthesis), *SREBP-1c* (transcription factor suppressed in FA, TG, and cholesterol synthesis), *FASN*, and *ACC-1* (acetyl-CoA carboxylase, a key enzyme for FA synthesis). As shown in Figure 4B, these four genes were upregulated by LXR stimulus (T090), which were significantly suppressed by BEs administration except LXR. Therefore, BEs suppress cellular FA synthetic pathway stimulated by LXR agonist. In case where the cells were stimulated by FA, BEs did not affect the expression levels of LXR, SREBP-1c, FASN, or ACC-1 (data not shown).

To elucidate the molecular mechanisms of BEs for autophagy, survival/proliferation, and antioxidant properties, we additionally examined the following protein expressions in non-steatotic AML12 cells (Figure 4C). p62/SQSTM1 protein was increased very mildly and transiently after the BEs treatment even in non-steatotic cells (day 3). This suggests that BEs did not directly affect p62/SQSTM1 expression in non-steatotic cells not expressing Rubicon (Figure 4C), and the increase of p62/SQSTM1 (Figure 4A) was secondary to reduction of Rubicon. Akt and STAT3 were both phosphorylated after the BEs treatment with slight increase of these protein expressions. Regarding the antioxidant proteins, the increase of protein expression was observed in MnSOD and catalase (Figure 4C).

Discussion

In the present study, we demonstrated that BEs improve fatty liver and the following liver injury using mouse liver cells and fatty liver model. We proposed the unique mechanisms of BEs' these effects on autophagy (Rubicon and p62/SQSTM1), lipid metabolism (FASN, STAT3), survival/proliferation (Akt/STAT3), and antioxidant (MnSOD/catalase).

We showed that BEs reduced the oxidative injury induced by hydrogen peroxide

(Figure 2C). BEs did not eliminate ROS directly (Figure 2D), but BEs induced the antioxidant proteins, MnSOD and catalase significantly (Figure 4C). Although Nrf2 is known to eventually confer resistance against oxidative stress to cells, significant activation of Nrf2 was not observed (data not shown). Though anthocyanin rich in BEs is known to suppress ROS production and prevent resulting toxic injury [35], it is not clear whether anthocyanin plays a central role for BEs' these effects.

We also showed that BEs may affect hepatocyte survival and/or proliferation. BEs improved cell viability in both non-steatotic and steatotic cells (Figures 1C, 2B, & 2C). As shown in Figure 4C, Akt and STAT3 seem to be both phosphorylated after the BEs treatment with slight increase of these protein expressions. The activation of these proteins by BEs should contribute to cell survival and proliferation in steatotic and non-steatotic cells. These data indicate that BEs may have pro-survival and regenerative potentials of tissues/organ in various conditions, though further investigation is required.

As we have already shown that STAT3 activation suppressed steatosis by inhibiting lipogenic genes such as SREBP-1c [36], a STAT3/SREBP pathway would definitely play an important role for the suppression of hepatic steatosis. Therefore, an activation of STAT3 may contribute at least partly to reduction of lipid accumulation by BEs.

We carefully examined the influence of BEs on fatty liver using both cultured liver cells and mouse liver. BEs suppressed lipid accumulation and its progression in both models (Figures 2A, 3B). BEs suppressed lipid accumulation in two different models (FA addition and TG synthesis) (Figures 4A, 4B) and enhanced lipolysis by autophagy (FA addition model) (Figure 4A). Most notable was Rubicon, which suppresses the late stage of autophagy and accelerates lipid accumulation [34]. It suppresses autophagosome fusion with lysosomes, which is the final step of autophagy. The rate of autophagy decreases with increasing intracellular Rubicon levels. Therefore, fatty liver may have progressed with increased hepatic Rubicon levels suppressing lipophagy. In the present study, we first report that BEs markedly

downregulate Rubicon and promote lipophagy (shown by an increase of p62/SQSTM1) in FA-induced steatotic hepatocytes [32,33]. BEs also reduced FASN expression, which is directly related to fat accumulation [31]. Therefore, BEs are predicted to suppress lipid accumulation in the liver by promoting lipolysis (decrease of Rubicon) and inhibiting TG synthesis (decrease of FASN). Because p62/SQSTM1 is also known to possess a cytoprotective effect [28], the increase of p62/SQSTM1 by BEs may also contribute to prevention against liver injury.

It has been reported that bilberry is antioxidant [5-8] and anti-inflammatory [37-39]. Also, in the present study, BEs showed antioxidant and anti-inflammatory properties other than defatting and protective property. This may suppress the steatosis-induced hepatic inflammation (hepatitis). As shown in Figure S2, BEs restrained biological effects against inflammatory cytokines such as TNF-α, IL-9, IL-1β and IFN-γ. This means that BEs have inhibitory effects against inflammation as well as defatting and protection, suggesting that BEs potentially prevent the progression from fatty liver to NASH. It is interesting to know that BEs may control these chemokine activities directly or indirectly, which may be important clinical targets of the future study of BEs.

Morrison *et al.* [40] comprehensively investigated the effects of Mirtoselect, an anthocyanin-rich bilberry extracts, on NASH and associated fibrosis. They examined the effects of anthocyanins-rich bilberry extracts on late pathology of NASH by animal experiment only. They concluded that Mirtoselect reduced development of NASH, attenuating both steatosis and inflammation as well as the development of hepatic fibrosis. On the other hand, we focused on the effect of BEs on earlier pathological events of fatty liver developing into NASH. We also studied its mechanism of action in detail by cell experiments, focusing on cell survival/proliferation, fat accumulation (incorporation, degradation), antioxidant, and inflammation. The results obtained from cell experiments were verified by animal experiments. Our study gives new mechanistic/therapeutic insights on the effect of bilberry

for the liver pathology developing into NASH.

Conclusion

Overall, we identified the factors that improve lipid accumulation, cytotoxicity, oxidative stress, and inflammation by BEs. Because hepatocyte fat accumulation, insulin resistance, oxidative stress, injury and sugar/fat metabolism are of particular importance in NASH progression, BEs was proved to target some of these crucial factors. We also showed that BEs potentially suppresses inflammation and fibrosis induced by fat accumulation and injury of liver. Based on the results detailed above, bilberry is expected to be a liver-friendly food supplement.

Author Contribution

M. O. designed and supervised the study. N. M., S. H. and M. O. wrote and revised the manuscript. S. H., Y, S. J. and H. Y. performed cell experiments. S. H. and M. O. performed animal experiments. S. H., T. S., N. M. and M. O. analyzed and discussed the results. All authors gave final approval and consented to be accountable on all matters.

Acknowledgment

We are particularly grateful for generous donation from Mr. & Mrs. Fujikawa (to M.O.).

Disclosure statement

The authors declare no conflict of interest.

Funding

This study was supported by the JSPS Grants-in-Aid for Scientific Researches (KAKENHI) [Grant Nos. 23659631 (to M.O.), 26670573 & 15H05659 (to S.H.)]. This work was also

supported in part by the Grants-in-Aid for the Regional R&D Proposal-Based Program from the Northern Advancement Center for Science & Technology (NOASTEC Foundation) (to M.O.), the Takeda Science Foundation (to S.H.), a research grant from the Akiyama Life Science Foundation (to S.H.).

References

[1] Chu W-k, Cheung SCM, Lau RAW, et al. Bilberry (*Vaccinium myrtillus* L.). In: Benzie IFE, Wachtel-Galor S, editors. Herbal Medicine: Biomolecular and Clinical Aspects. Florida (FL): CRC Press; 2011. p. 55-71.

[2] Ferlemi AV, Lamari FN. Berry leaves: An alternative source of bioactive natural products of nutritional and medicinal value. Antioxidants 2016;5:17.

[3] Akerström A, Jaakola L, Bång U, et al. Effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of *Vaccinium myrtillus* L. (bilberries). J Agric Food Chem. 2010;58:11939-11945.

[4] Burdulis D, Sarkinas A, Jasutiené I, et al. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. Acta Pol Pharm. 2009;66:399-408.

[5] Erlund I, Marniemi J, Hakala P, et al. Consumption of black currants, lingonberries and bilberries increases serum quercetin concentrations. Eur J Clin Nutr. 2003;57:37-42.

[6] Matsunaga N, Tsuruma K, Shimazawa M, et al. Inhibitory actions of bilberry anthocyanidins on angiogenesis. Phytother Res. 2010;24:S42-S47.

[7] Viljanen K, Kylli P, Kivikari R, et al. Inhibition of protein and lipid oxidation in liposomes by berry phenolics. J Agric Food Chem. 2004;52:7419-7424.

[8] Yao Y, Vieira A. Protective activities of *Vaccinium* antioxidants with potential relevance to mitochondrial dysfunction and neurotoxicity. Neurotoxicology 2007;28:93-100.

[9] Mian E, Curri SB, Lietti A, et al. Anthocyanosides and the walls of the microvessels:

395 further aspects of the mechanism of action of their protective effect in syndromes due to
396 abnormal capillary fragility. Minerva Med. 1977;68:3565-3581.
397 [10] Bottecchia D, Bettini V, Martino R, et al. Preliminary report on the inhibitory effect of
398 *Vaccinium myrtillus* anthocyanosides on platelet aggregation and clot retraction.
399 Fitoterapia 1987;58:3-8.
400 [11] Harvsteen, B. Flavonoids, a class of natural products of high pharmacological potency.
401 Biochem Pharmacol. 1983;32:1141-1148.
402 [12] Gabor, M. Pharmacologic effects of flavonoids on blood vessels. Angiologica 1972;9:
403 355-374.
404 [13] Tumbasa V, Čanadanović-Brunet J, Gille L, et al. Superoxide anion radical scavenging
405 activity of bilberry (*Vaccinium myrtillus* L.). J Berry Res. 2010;1:13-23.
406 [14] Valentová K, Ulrichová J, Cvak L, et al. Cytoprotective effect of a bilberry extract
407 against oxidative damage of rat hepatocytes. Food Chem. 2007;101:912-917.
408 [15] Lankinen M, Schwab U, Kolehmainen M, et al. Whole grain products, fish and bilberries
409 alter glucose and lipid metabolism in a randomized, controlled trial: The sysdimet study.
410 PLoS One 2011;6:e22646.
411 [16] Lehtonen HM, Suomela JP, Tahvonen R, et al. Different berries and berry fractions have
412 various but slightly positive effects on the associated variables of metabolic diseases on
413 overweight and obese women. Eur J Clin Nutr. 2011;65:394-401.
414 [17] Mauray A, Felgines C, Morand C, et al. Nutrigenomic analysis of the protective effects
415 of bilberry anthocyanin-rich extract in apo E-deficient mice. Genes Nutr.
416 2010;5:343-353.
417 [18] Mykkänen OT, Huotari A, Herzig K-H, et al. Wild blueberries (*Vaccinium myrtillus*)
418 alleviate inflammation and hypertension associated with developing obesity in mice fed
419 with a high-fat diet. PLoS One 2014;9:e114790.
420 [19] Persson IA, Persson K, Andersson RG. Effect of *Vaccinium myrtillus* and its polyphenols

421 on angiotensin-converting enzyme activity in human endothelial cells. J Agric Food
422 Chem. 2009;57:4626-4629.
423 [20] Suzuki R, Tanaka M, Takanashi M, et al. Anthocyanidins-enriched bilberry extracts
424 inhibit 3T3-L1 adipocyte differentiation via the insulin pathway. Nutr Metab. 2011;8:14.
425 [21] Takikawa M, Inoue S, Horio F, et al. Dietary anthocyanin-rich bilberry extract
426 ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated
427 protein kinase in diabetic mice. J Nutr. 2010;140:527-533.
428 [22] Ziberna L, Lunder M, Tramer F, et al. The endothelial plasma membrane transporter
429 bilitranslocase mediates rat aortic vasodilation induced by anthocyanins. Nutr Metab
430 Cardiovasc Dis. 2013;23:68-74.
431 [23] Marra F, Svegliati-Baroni G. Lipotoxicity and the gut-liver axis in NASH pathogenesis. J
432 Hepatol. 2018;68:280-295.
433 [24] Rinella ME. Nonalcoholic fatty liver disease: a systematic review. JAMA
434 2015;313:2263-2273.
435 [25] Cha J-Y, Repa JJ. The liver X receptor (LXR) and hepatic lipogenesis. The
436 carbohydrate-response element-binding protein is a target gene of LXR. J Biol Chem.
437 2007;282:743-751.
438 [26] Nagrath D, Xu H, Tanimura Y, et al. Metabolic preconditioning of donor organs:
439 defatting fatty livers by normothermic perfusion *ex vivo*. Metab Eng. 2009;11:274-283.
440 [27] Nativ NI, Yarmush G, So A, et al. Elevated sensitivity of macrosteatotic hepatocytes to
441 hypoxia/reoxygenation stress is reversed by a novel defatting protocol. Liver Transplant.
442 2014;20:1000-1011.
443 [28] Haga S, Yimin, Ozaki M. Relevance of FXR-p62/SQSTM1 pathway for survival and
444 protection of mouse hepatocytes and liver, especially with steatosis. BMC Gastroenterol.
445 2017;17:9.
446 [29] Haga S, Remington SJ, Morita N, et al. Hepatic ischemia induced immediate oxidative

447 stress after reperfusion and determined the severity of the reperfusion-induced damage.
448 Antioxid Redox Signal. 2009;11:2563-2572.
449 [30] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
450 quantitative PCR and the $2^{\Delta\Delta C}{}_{T}$ Method. Methods 2001;25:402-408.
451 [31] Dorn, C., Riener, M. O., Kirovski, G., et al. Expression of fatty acid synthase in
452 nonalcoholic fatty liver disease. Int J Clin Exp Pathol. 2010;3:505-514.
453 [32] Haga S, Ozawa T, Yamada Y, et al. p62/SQSTM1 plays a protective role in oxidative
454 injury of steatotic liver in a mouse hepatectomy model. Antioxid Redox Signal.
455 2014;21:2515-2530.
456 [33] Park JS, Oh SY, Lee DH, et al. p62/SQSTM1 is required for the protection against
457 endoplasmic reticulum stress-induced apoptotic cell death. Free Rad Res.
458 2016:50:1408-1421.
459 [34] Tanaka S., Hikita H., Tatsumi T., et al. Rubicon inhibits autophagy and accelerates
460 hepatocyte apoptosis and lipid accumulation in nonalcoholic fatty liver disease in mice.
461 Hepatology 2016;64:1994-2014.
462 [35] Pandey KB, Rizv SI. Plant polyphenols as dietary antioxidants in human health and
463 disease. Oxid Med Cell Longev. 2009;2:270-278.
464 [36] Inoue H, Ogawa W, Ozaki M, et al. Role of STAT-3 in regulation of hepatic
465 gluconeogenic genes and carbohydrate metabolism *in vivo*. Nat. Med. 2004;10:168-174.
466 [37] De Mello VD, Schwab U, Kolehmainen M, et al. A diet high in fatty fish, bilberries and
467 wholegrain products improves markers of endothelial function and inflammation in
468 individuals with impaired glucose metabolism in a randomised controlled trial: The
469 sysdimet study. Diabetologia 2011;54:2755-2767.
470 [38] Domitrović R, Jakovac H. Effects of standardized bilberry fruit extract (Mirtoselect®) on
471 resolution of CCl_4-induced liver fibrosis in mice. Food Chem Toxicol.
472 2011;49:848-854.

[39] Miyake S, Takahashi N, Sasaki M, et al. Vision preservation during retinal inflammation by anthocyanin-rich bilberry extract: cellular and molecular mechanism. Lab Investig 2012;92:102-109.

[40] Morrison MC, Liang W, Mulder P, et al. Mirtoselect, an anthocyanin-rich bilberry extract, attenuates non-alcoholic steatohepatitis and associated fibrosis in ApoE* 3Leiden mice. J Hepatol. 2015;62:1180-1186.

Table 1. Primer list for the quantitative real-time PCR (qRT-PCR).

Symbol	Gene name	Primer sequence
LXR	Liver X receptor α	F: 5’-CTGGGGTTGCTTTAGGGATAG-3’ R: 5’-TTCCGCTTTTGTGGACGAAG-3’
SREBP-1c	Sterol regulatory element-binding protein-1c	F: 5’-AAGCTCAAGCAGGAGAACC-3’ R: 5’-ATGCCCTCCATAGACACATC-3’
FASN	Fatty acid synthase	F: 5’-GGGCTCTATGGATTACCC-3’ R: 5’-AAAAGGAGGCGTCGAAC-3’
ACC-1	Acetyl-CoA carboxylase	F: 5’-GGCTGCTAAGGTGGAAGTAGG-3’ R: 5’-TGCCTGATGATCGCACGAAC-3”
36B4	Acidic ribosomal phosphoprotein P0	F: 5’-ATGGGTACAAGCGCGTCCTG-3” R: 5’-GCCTTGACCTTTTCAGTAAG-3’

F, forward; R, reverse

Table 2. Body weight and food intake in mice fed a high-fat and high-cholesterol (HF&HC) diet with/without bilberry fruits extracts (BEs) for 8 weeks. Data are means ± SEM; n = 4-8 per group. *$p < 0.05$ vs. HF&HC group. There were no significant differences in initial body weight or food intake between groups.

	HF&HC	5% BEs + HF&HC	10% BEs + HF&HC
Initial body weight (g)	37.58 ± 0.80	38.60 ± 0.74	39.15 ± 0.48
Final body weight (g)	49.83 ± 1.01	46.37 ± 1.60	44.43 ± 1.17*
Food intake (g/d)	3.89 ± 0.13	3.49 ± 0.10	3.21 ± 0.10

Figure legends

Figure 1. Bilberry fruits extracts (BEs) improved steatotic hepatocyte growth (survival/proliferation) (AML12 cells). (A) Administration of free fatty acids (FA) consisting of 100 μM oleic and linoleic acids or 1 μM T0901317 (T090, an agonist for LXRα) induced substantial lipid deposition in murine AML12 hepatocytes (Day 5). Right panel: lipids in cytosol and nuclei stained with AdipoRed (green) and Hoechst33342 (red, pseudo-color), respectively. Scale bar: 50 μm. Different letters indicate statistically significant differences among groups ($p<0.05$); n = 4; mean ± SEM. (B) Cell growth deteriorated similarly in both FA- and T090-induced steatotic hepatocytes. The same letter or no letter indicates that the difference is not significant within each time-point group ($p<0.05$); n = 3-4; mean ± SEM). (C) BEs promoted cell growth of steatotic hepatocytes induced by FA- and T090-administration (left and right panels, respectively) with a peak concentration at 5 μg/mL. Since no significant difference in cell growth was observed between 12 hours and 48 hours, only the data after 60 hours are shown. The same letter or no letter indicates that the difference is not significant within each time-point group ($p<0.05$); n = 3-4; mean ± SEM.

Figure 2. Bilberry fruits extracts (BEs) reduced lipid deposition in steatotic hepatocytes, promoted cell growth, and conferred resistance to oxidative stress. (A) BEs significantly suppressed lipid accumulation in hepatocytes. Right panel: lipids in cytosol and nuclei in green (AdipoRed) and red (Hoechst33342, pseudo-color), respectively. Scale bar: 50 μm. Different letters indicate statistically significant differences among groups ($p<0.05$); n = 4-6; mean ± SEM. (B) BEs promoted cell growth at peak concentrations of 1-5 μg/mL in non-steatotic hepatocytes. The same letter indicates that the difference is not significant ($p\geq0.05$); n=3-6). (C) BEs conferred resistance to a 24-h H_2O_2 challenge (500 μM) in hepatocytes. Lower panel: more live cells were observed in BEs-treated hepatocytes (1

μg/mL) than untreated liver cells. Scale: 50 μm. The same letter indicates that the difference is not significant ($p \geq 0.05$); n=3-6). (D) BEs did not directly react with and/or scavenge cellular ROS (left panel; n = 8).

Figure 3. Bilberry fruits extracts (BEs) improved liver steatosis, liver damage, and hyperglycemia in mice fed HF&HC diets. (A) HF&HC diet (8 weeks) induced liver mass enlargement with no increase in body weight. The same letter indicates that the difference is not significant ($p \geq 0.05$); n=4. (B) BEs improved hepatic steatosis. Scale bar: 100 μm. Hepatic TG levels were reduced in BEs-treated livers (right panel). The same letter indicates that the difference is not significant ($p \geq 0.05$); n=4. (C) Fibrosis stained blue with Azan and was observed in the periportal area (arrowhead) of HF&HC-treated livers. (D) Blood biochemistry revealed that BEs improved steatosis-induced liver damage (ALT and AST) and hypercholesterolemia (total cholesterol, T-CHO). The same letter indicates that the difference is not significant within each group ($p \geq 0.05$); n = 4. Mean ± SEM.

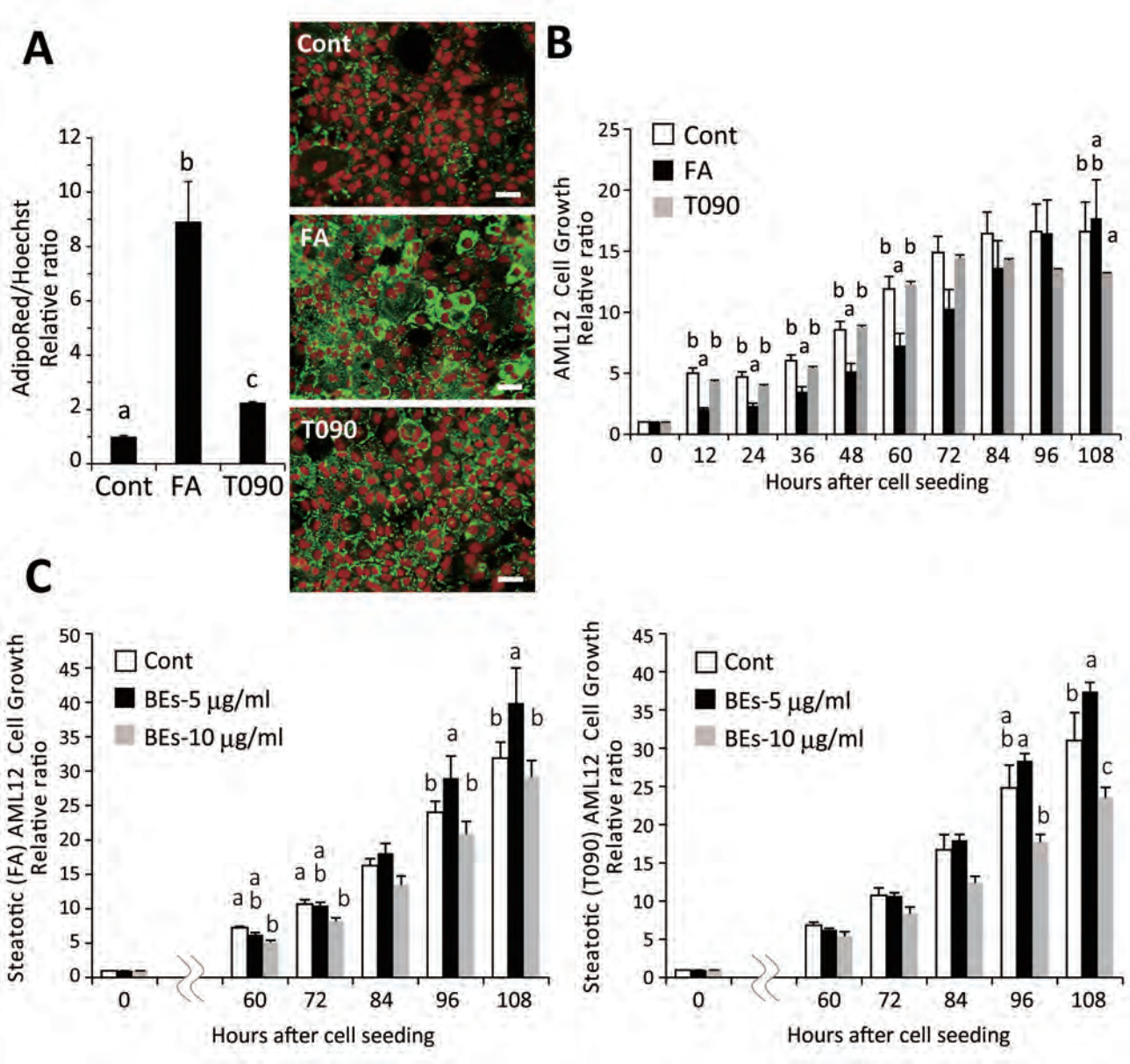
Figure 4. Molecular analyses of the effects of bilberry fruits extracts (BEs) on murine AML12 hepatocytes. (A) Effects of BEs on autophagy (lipophagy) (Rubicon, p62/SQSTM1) and fatty acid (FA) synthesis (fatty acid synthase, FASN) were examined in FA- / T0901317 (T090; LXRα agonist)-treated AML12 cells by western blot. Each blot represents ≥3 independent experiments. The duplicates of immunoblots are taken from the specimens of experiments performed at different times. ImageJ (NIH, Bethesda, MD, USA) was used for the quantitative analysis of western blot. The same letter indicates that the difference is not significant among groups ($p \geq 0.05$). (B) Effects of BEs on FA synthesis were examined by qRT-PCR. The same letter indicates that the difference is not significant among groups ($p \geq 0.05$). (C) Levels of proteins associated with autophagy, survival/proliferation, and

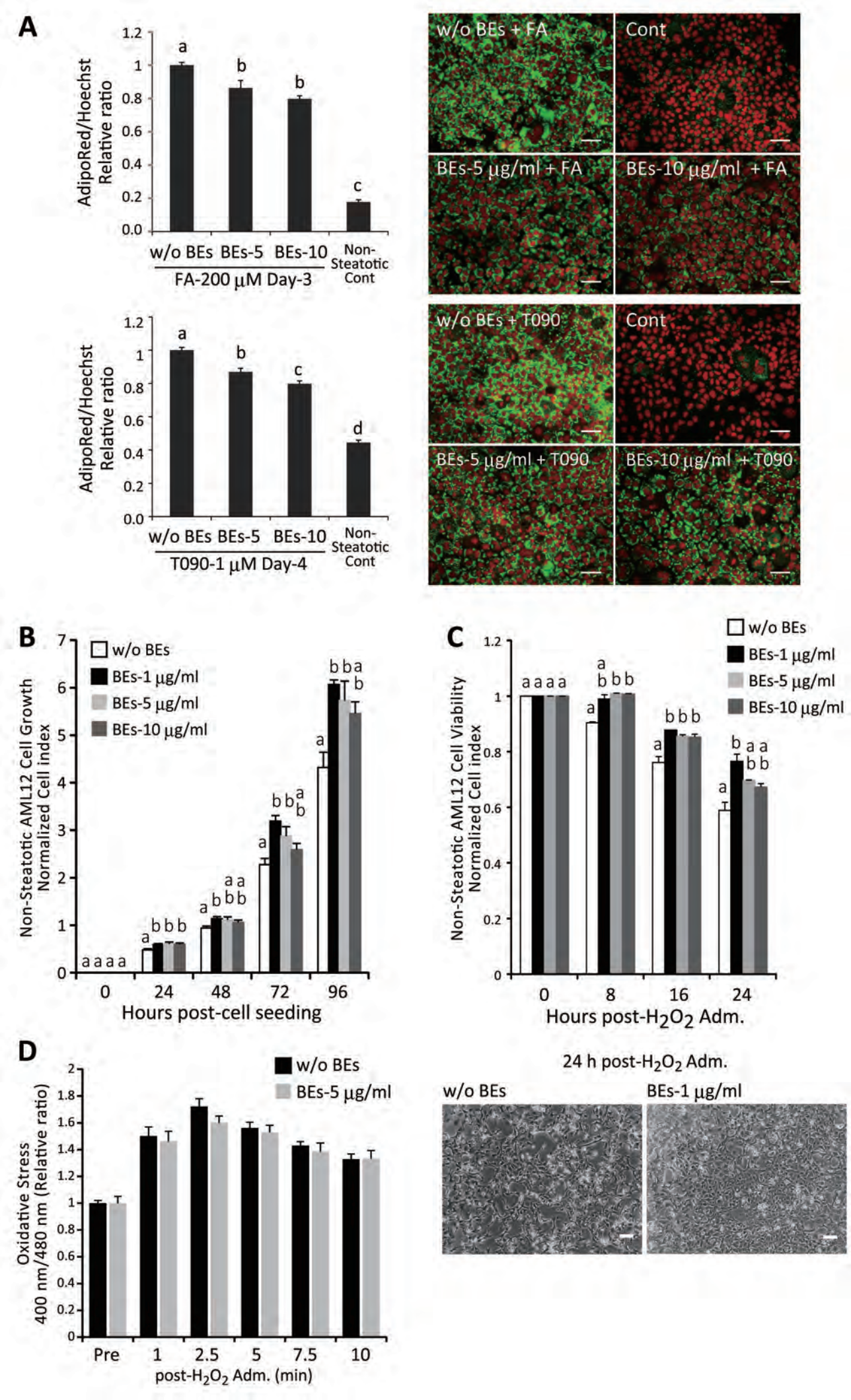
antioxidant properties were slightly increased by the addition of BEs in non-steatotic AML12 cells (Day 3 and/or Day 7). Each blot represents ≥3 independent experiments. Each experiment was performed 3× and the data are expressed as means ± SEM (A, B, and C). The same letter or no letter indicates that the difference is not significant within each time-point group ($p \geq 0.05$).

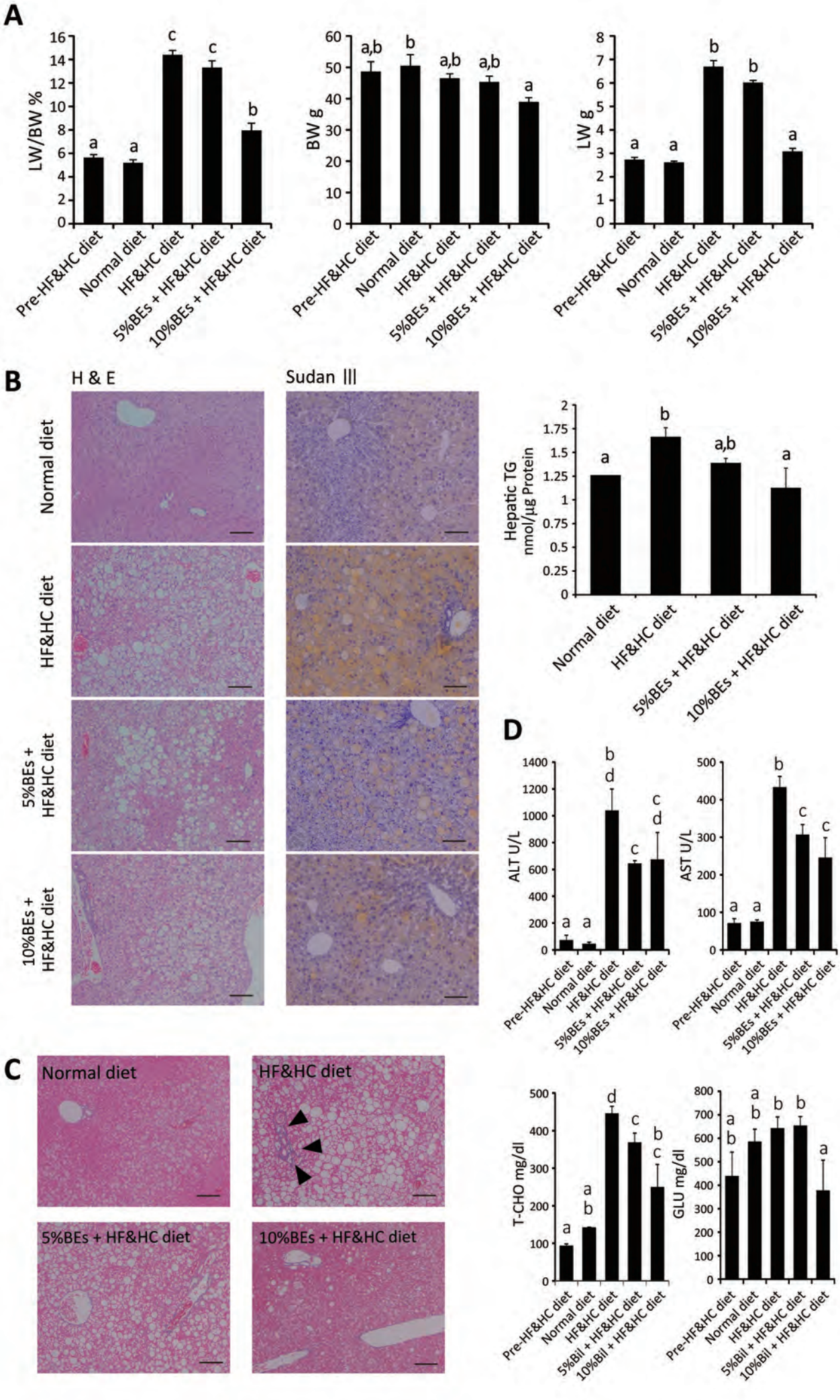
Supplemental data

Figure S1. Cytotoxicity of bilberry fruits extracts (BEs) against AML12 cells estimated with LDH assay. Values are expressed as means ± SEM (n = 2).

Figure S2. Levels of pro-inflammatory cytokines induced by the high-fat and high-cholesterol (HF&HC) diet decreased with bilberry fruits extracts (BEs) feeding. Pro-inflammatory cytokines (Interleukin-1β (IL-1β), IL-9, IL-12, Interferon γ (IFNγ), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor α (TNFα)) in mouse plasma were measured with a Bio-Plex Multiplex System (Bio-Rad Laboratories, Hercules, CA, USA). Values are expressed as means ± SEM (n = 2-3). There were no significant differences between the HF&HC diet group and the BEs + HF&HC diet group.


A
Cont
FA
T090
AdipoRed/Hoechst Relative ratio
Cont FA T090
B
Cont
FA
T090
AML12 Cell Growth Relative ratio
Hours after cell seeding
C
Cont
BEs-5 µg/ml
BEs-10 µg/ml
Steatotic (FA) AML12 Cell Growth Relative ratio
Hours after cell seeding
Steatotic (T090) AML12 Cell Growth Relative ratio
Hours after cell seeding


A
AdipoRed/Hoechst Relative ratio
w/o BEs
BEs-5
BEs-10
Non-Steatotic Cont
FA-200 μM Day-3
T090-1 μM Day-4
w/o BEs + FA
Cont
BEs-5 μg/ml + FA
BEs-10 μg/ml + FA
w/o BEs + T090
BEs-5 μg/ml + T090
BEs-10 μg/ml + T090
B
Non-Steatotic AML12 Cell Growth Normalized Cell index
w/o BEs
BEs-1 μg/ml
BEs-5 μg/ml
BEs-10 μg/ml
Hours post-cell seeding
C
Non-Steatotic AML12 Cell Viability Normalized Cell index
Hours post-H2O2 Adm.
D
Oxidative Stress 400 nm/480 nm (Relative ratio)
post-H2O2 Adm. (min)
24 h post-H2O2 Adm.


A
LW/BW %
BW g
LW g
Pre-HF&HC diet
Normal diet
HF&HC diet
5%BEs + HF&HC diet
10%BEs + HF&HC diet
B
H & E
Sudan III
Normal diet
HF&HC diet
5%BEs + HF&HC diet
10%BEs + HF&HC diet
Hepatic TG nmol/μg Protein
C
Normal diet
HF&HC diet
5%BEs + HF&HC diet
10%BEs + HF&HC diet
D
ALT U/L
AST U/L
T-CHO mg/dl
GLU mg/dl
Pre-HF&HC diet
Normal diet
HF&HC diet
5%Bil + HF&HC diet
10%Bil + HF&HC diet

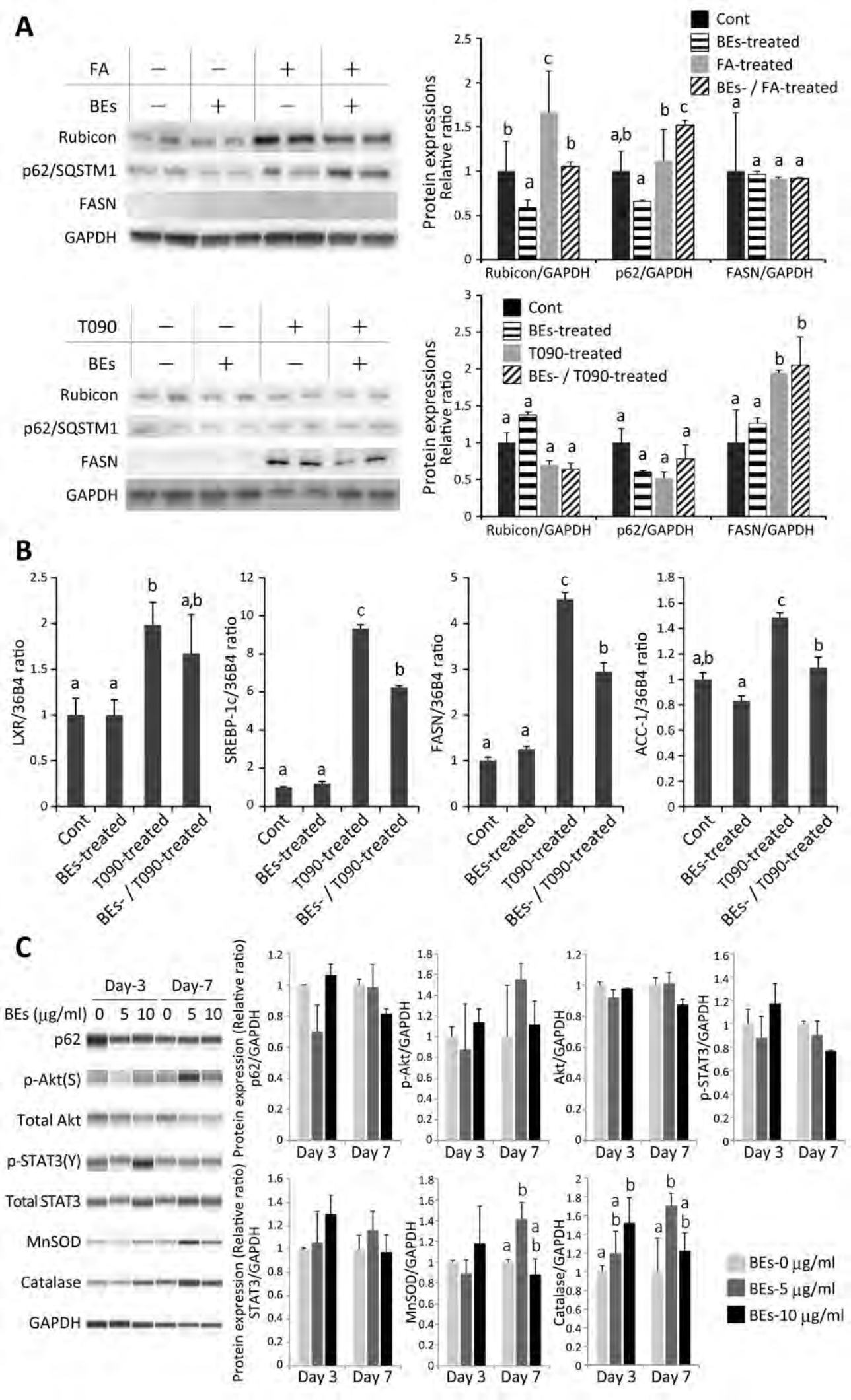

A
FA
BEs
Rubicon
p62/SQSTM1
FASN
GAPDH
Cont
BEs-treated
FA-treated
BEs- / FA-treated
Protein expressions Relative ratio
Rubicon/GAPDH
p62/GAPDH
FASN/GAPDH
T090
T090-treated
BEs- / T090-treated
B
LXR/36B4 ratio
SREBP-1c/36B4 ratio
FASN/36B4 ratio
ACC-1/36B4 ratio
C
Day-3
Day-7
BEs (μg/ml) 0 5 10 0 5 10
p62
p-Akt(S)
Total Akt
p-STAT3(Y)
Total STAT3
MnSOD
Catalase
GAPDH
Protein expression (Relative ratio) p62/GAPDH
p-Akt/GAPDH
Akt/GAPDH
p-STAT3/GAPDH
Protein expression (Relative ratio) STAT3/GAPDH
MnSOD/GAPDH
Catalase/GAPDH
Day 3
Day 7
BEs-0 μg/ml
BEs-5 μg/ml
BEs-10 μg/ml

Figure S1

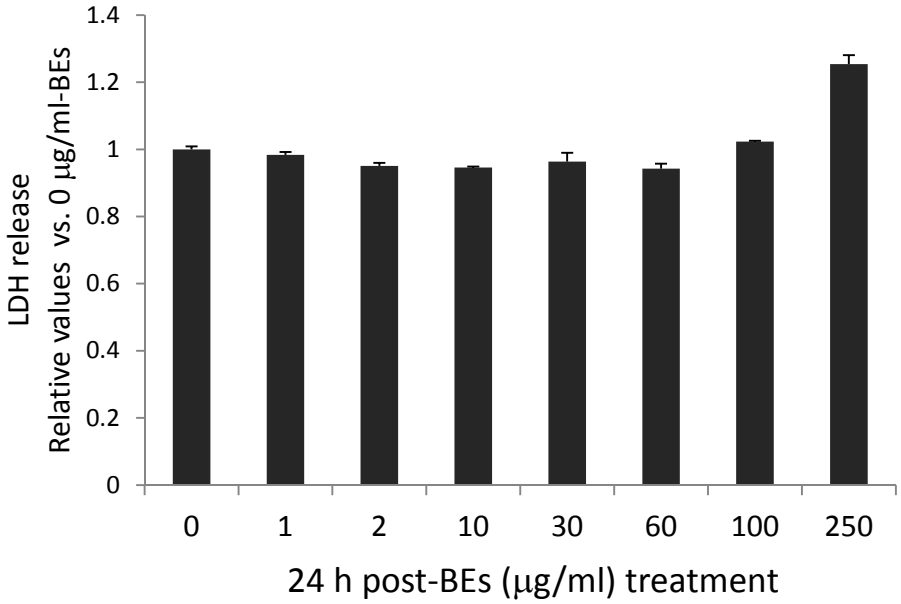
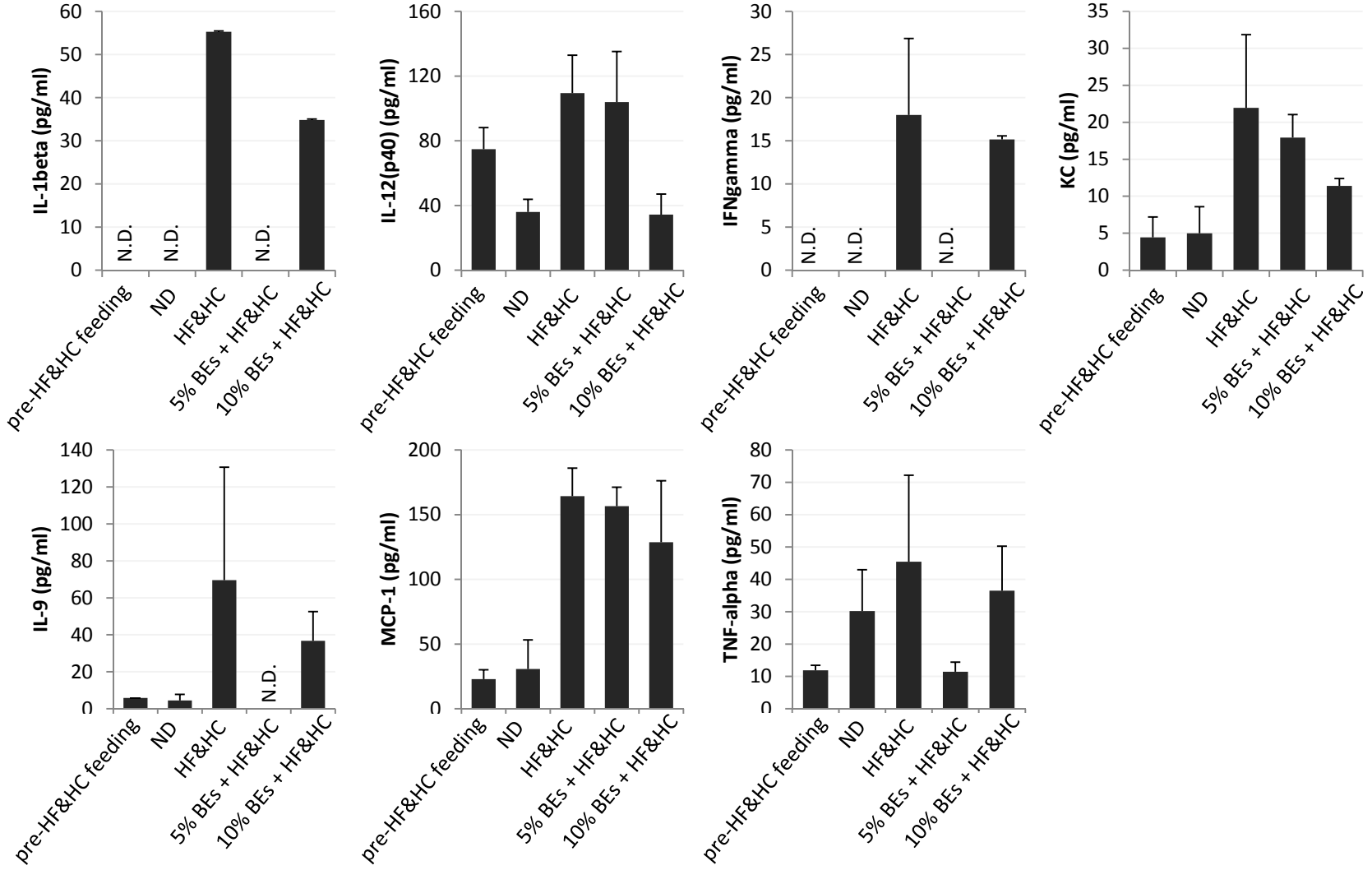

LDH release
Relative values vs. 0 μg/ml-BEs
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
10
30
60
100
250
24 h post-BEs (μg/ml) treatment

Figure S2


IL-1beta (pg/ml)
N.D.
N.D.
N.D.
IL-12(p40) (pg/ml)
IFNgamma (pg/ml)
N.D.
N.D.
N.D.
KC (pg/ml)
IL-9 (pg/ml)
N.D.
MCP-1 (pg/ml)
TNF-alpha (pg/ml)
pre-HF&HC feeding
ND
HF&HC
5% BEs + HF&HC
10% BEs + HF&HC